Chapter 4

Gene Editing and Protein Tagging in the Oomycete *Phytophthora infestans* Using CRISPR-Cas12a

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Abstract

Molecular genetic tools such as CRISPR-Cas gene editing systems are invaluable for understanding gene and protein function and revealing the details of a pathogen's life and disease cycles. Here we present protocols for genome editing in *Phytophthora infestans*, an oomycete with global importance as a pathogen of potato and tomato. Using a vector system that expresses variants of Cas12a from *Lachnospiraceae bacterium* and its guide RNA from a unified transcript, we first present a method for editing genes through the non-homologous end-joining (NHEJ) pathway. We then describe an application of homology-directed repair (HDR), in which Cas12a is used to fuse a protein-coding gene with a fluorescent or epitope tag. Both methods should be adaptable to many oomycetes other than *P. infestans*.

Key words Oomycete, Gene editing, CRISPR, Transformation, Fluorescent reporter

1 Introduction

Methods for modifying genomes derived from bacterial CRISPR systems are becoming indispensable for research in many biological systems [1]. CRISPR is already accelerating studies of several members of the oomycete genus *Phytophthora* [2–5], which includes some of the world's most destructive plant pathogens [6]. For example, the focus of this chapter is the globally significant potato and tomato pathogen *Phytophthora infestans* [7]. In combination with genome sequence and transcriptome data [8, 9], and other tools for genome manipulation such as DNA-mediated transformation, homology-based gene silencing, and systems for expressing fluorescently labeled proteins [10–13], the expanding toolkit for *Phytophthora* holds promise for illuminating its biology and helping to identify strategies for controlling plant disease.

The CRISPR tools used most frequently for studying bacteria and eukaryotes employ either the Cas9 or Cas12a (Cpf1) proteins [1]. Guided by RNA sequences targeting a genomic locus, both

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cause double-stranded breaks in the host chromosome. These are repaired by the host's non-homologous end-joining (NHEJ) pathway or, in the presence of a template, by homology-directed repair (HDR) [14]. Repair by NHEJ usually involves the removal or addition of several bases, often leading to a change in reading frame and a defective protein. The cellular role of the affected gene can then be assessed through phenotypic analysis. While HDR can also be used to knock out expression of a functional protein, its other applications include gene replacement, promoter modification, tagging genes with an epitope or fluorescent marker, or inserting DNA at a "safe harbor" to avoid affecting endogenous genes or obtaining consistent expression of a reporter or other transgene [15–17].

Cas9 and Cas12a differ in several important aspects. Each involves a protospacer adjacent motif (PAM), which is a short DNA sequence found near the DNA site that is targeted for cleavage. However, the PAM for the commonly used form of Cas9 is 5′-NGG while for Cas12a this is 5′-TTTV [18]. Another difference is that Cas12a, particularly from *Lachnospiraceae bacterium*, retains activity at lower temperatures to a greater extent than Cas9 [19]. This makes it attractive for use in ectothermic animals, plants, and many species of *Phytophthora*. Another difference is that CRISPR RNA precursors used for editing with Cas12a are much shorter than those employed by Cas9, since Cas12a can process its precursor directly without the trans-activating RNA scaffold required by Cas9 [20]. This makes synthesizing a Cas12a guide RNA (gRNA, also known as crRNA) less expensive and facilitates the construction of multiplex editing arrays [21]. Cas12a is also reported to generate larger deletions, which may increase the rate of loss-of-function events, and to have fewer-off target effects [22]. Despite these advantages of Cas12a, having both systems could be useful since their distinct PAM sequences increase the number of potentially editable sites.

Initial attempts to employ CRISPR-Cas9 in *P. infestans* failed due to the presumed toxicity of the enzyme, a situation which is not unique to oomycetes [23]. Instead, we developed a successful CRISPR-Cas12a system [3]. Unlike the Cas9 method deployed in other species of *Phytophthora* in which the editing protein and gRNA are encoded by separate genes [2], ours produces the nuclease and guide RNA in a single transcript to increase the likelihood that both would be expressed in a transformant. Another difference is that we make use of the innate RNase activity of Cas12a to form the mature guide RNA, instead of ribozyme sequences which appeared to function less effectively in *P. infestans* [3].

This chapter provides protocols for two distinct applications of Cas12a. First, we describe gene editing through NHEJ. Second, we illustrate the use of HDR to tag a protein, by expressing Cas12a and a guide RNA in the presence of a repair template encoding a

fluorescent protein. The resulting fusion can be visualized by microscopy to investigate the function of the protein or other aspects of cellular dynamics. While our protocols focus on *P. infestans*, they should be adaptable to other members of the *Phytophthora* genus or close relatives such as *Pythium* species.

2 Materials

2.1 Vector Construction

1. Cas12a editing vector (pSTU1 or pSTUC1; *see* **Note 1**).
2. Upper and lower-strand oligonucleotides for constructing cassettes encoding gRNAs (see Subheading 3.1 for design instructions).
3. 42 °C heating block or water bath.
4. Microcentrifuge.
5. Thermal cycler.
6. Chemically competent (or electrocompetent) *Escherichia coli*, such as strain DH5α.
7. Lysogeny Broth agar (LB): 1.0% NaCl, 1.0% tryptone, 0.5% yeast extract, 1.5% agar.
8. Terrific Broth (TB): Combine 24 g yeast extract, 12 g tryptone, and 4 g glycerol in water to a total volume of 900 mL. Separately, mix 2.3 g KH2PO4 with 16.4 g M K2HPO4 in water to a total volume of 100 mL. Autoclave both solutions separately and then combine.
9. Super Optimal Catabolite broth (SOC): 2% tryptone, 0.5% yeast extract, 0.05% NaCl.
10. T4 DNA ligase and 10× buffer for T4 DNA ligase (including ATP).
11. 0.5 M NaCl.
12. BsaI restriction enzyme and buffer (see **Note 2**).
13. Small- and large-scale plasmid purification kits.
14. Primer 3′HAM34-R (5′-CGCCATCACCGATTCGTC- 3′).
15. For HDR, a general vector such as pBluescript for cloning the donor DNA.

2.2 Transformation of Protoplasts

1. Room temperature centrifuge with swinging-bucket rotor.
2. Orbital shaker capable of slow speeds (<50 rpm).
3. Hemocytometer.
4. Syringe filters, preferably low protein-binding with prefilter.
5. 50–70 μm nylon mesh or cell strainer.
6. Sterile glassware, including 1 L glass flasks and 400 mL beakers.

7. Forceps, preferably with an angled tip.
8. Sterile spreaders, either disposable plastic or made from a bent glass rod.
9. A well-sporulating diploid strain of *Phytophthora infestans*.
10. Rye-sucrose broth: For 1 L, soak 60 g clean rye seeds (preferably organic) in 0.5 L water at room temperature for 24 h. The mixture should smell "sweet" and become just slightly acidic, with a pH of about 6.0. Then, break the grains into smaller pieces (about 1/4 the size of a whole grain) using a blender, and autoclave for 30 min. After removing large grain pieces using a colander, add water to 1 L and 20 g sucrose. We recommend storing frozen aliquots which are sterilized before use.
11. Amended lima bean (ALBA) media: 5 g/L sorbitol, 5 g/L mannitol, 5 g/L dextrose, 3 g/L KNO_3, 1 g/L K_2HPO_4, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.1 g/L $CaCl_2$, 2 g/L yeast extract, 2 mL vitamin stock, 2 mL trace elements, and 250 mL lima bean extract (prepared by blending and then autoclaving 280 g/L lima beans in water, then clarified by centrifugation). The vitamin stock (stored in the dark) contains 0.2 mg biotin, 0.2 mg folic acid, 12 mg I-inositol, 60 mg nicotinic acid, 18 mg pyridoxine-HCl, 15 mg riboflavin, 38 mg thiamine-HCl, and 50 mL coconut milk in 300 mL water. The trace element stock contains 215 mg $FeC_6H_5O_7.3H_2O$, 150 mg $ZnSO_4.7H_2O$, 30 mg $CuSO_4.5H_2O$, 10 mg $MnSO_4.H_2O$, 10 mg H_3BO_3, and 7 mg MoO_3 in 400 ml. This medium is stored at 4 °C. If a precipitate is seen in the ALBA after refrigeration, clarify by centrifugation for 15 min at 5000 × *g*.
12. Protoplasting enzymes: Cellulase and β-glucanase (*see* **Note 3**).
13. Protoplasting buffer (FPB): 0.4 M mannitol, 20 mM KCl, 20 mM MES pH 5.7, 10 mM $CaCl_2$.
14. Mannitol-Tris (MT): 1 M mannitol, 10 mM Tris pH 7.5.
15. Mannitol-Tris-Calcium (MTC): MT containing 10 mM $CaCl_2$.
16. Polyethylene glycol mix: 50% PEG-MW 3350 (Millipore-Sigma) containing 25 mM $CaCI_2$ and 10 mM Tris 7.5.
17. Lipofectin (Thermo Fisher Scientific; optional, *see* **Note 4**).
18. Protoplast regeneration media: rye-sucrose broth with 1 M mannitol, clarified by centrifugation for 15 min at 5000× *g*.
19. Selective media: rye-sucrose containing 1.5% agar and 8 μg/mL G-418 (*see* **Note 5**).

2.3 Detecting Editing

1. Boiling water bath.
2. Thermal cycler.
3. DNA extraction buffer: 0.2 M Tris pH 8.5, 0.25 M NaCl, 25 mM EDTA, 2% sodium dodecyl sulfate.
4. 1:1 phenol:chloroform.
5. 100% isopropanol.
6. 70% ethanol.
7. Low TE: 10 mM Tris pH 7.9, 0.1 mM EDTA.
8. Polymerase chain reaction (PCR) reagents: This can be purchased as a premade 2× mix or assembled from individual components. A 1× reaction would contain 50 mM KCl, 10 mM Tris-Cl pH 8.3, 1 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate (dNTP) stock, and 1 unit *Taq* polymerase per 25 μL, plus primers.
9. PCR primers to screen for editing. These are usually designed to bind about 75 nt on either side of the site targeted for editing.
10. Kits or reagents for cleaning up samples prior to sequencing (*see* **Note 6**).

3 Methods

3.1 Designing Guide RNAs for Editing by NHEJ

1. We recommend using the browser-based programs EuPaGDT or DeepCpf1 to identify the gRNA [24, 25]. For EuPaGDT, select the Cpf1/Cas12a nuclease option, TTTV for the PAM, and 23 nt for the guide length. V indicates A, C, or G, but not T. Note that the gRNA will include the 23 nt downstream (3′ end) of the PAM, but not the TTTV itself (Fig. 1a). When evaluating gRNA candidates, they should have 35 to 65% GC and little to no secondary structure with no more than 3 consecutive paired bases [1]. For knocking out protein function, gRNAs close to the 5′ end of the coding sequences or upstream of a functional domain are preferred to maximize the likelihood that editing will prevent formation of an active protein.
2. If possible, select several gRNAs to maximize the probability of editing. These could be tested in separate plasmids, but we usually construct arrays of two to three gRNAs in a single plasmid. We prefer to select targets within 500 nt of each other, so that a single set of primers can be used for PCR and sequence analysis of each potential editing site.
3. Consider using chromatin accessibility data to prioritize gRNA candidates, since studies in other taxa have indicated that nucleosome-free regions are more editable [26].

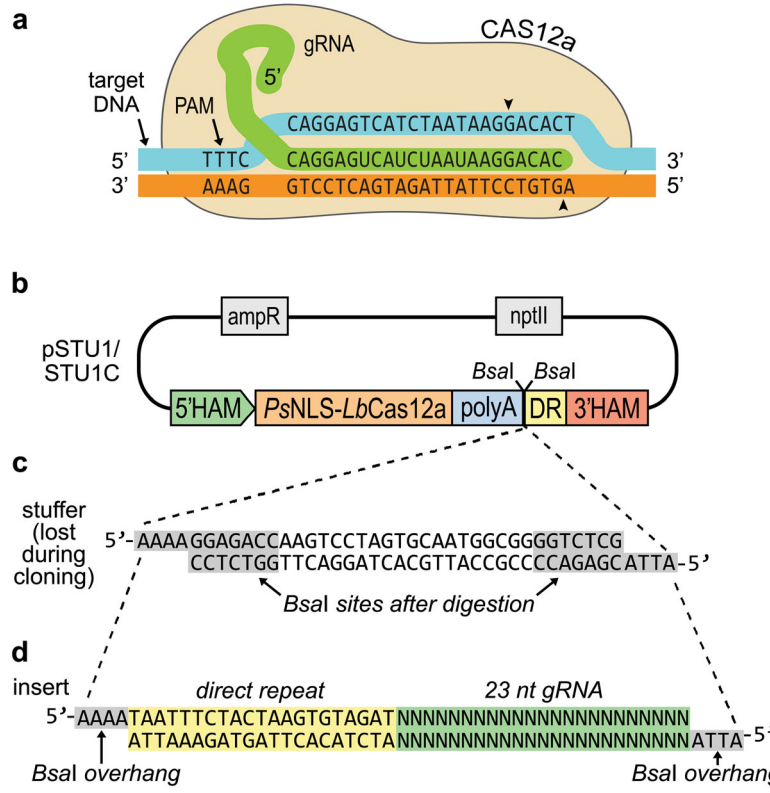

Fig. 1 (**a**) Target digestion by Cas12a. Labeled are a representative gRNA and PAM in host DNA. It should be noted that the orientation of the PAM relative to the gRNA is different for Cas9 and Cas12a. Arrowheads mark the staggered cuts made by Cas12a; these sites are approximations, since Cas12 often cuts at several positions [38]. (**b**) Schematic of Cas12a plasmids. Shown are the *Ham34* transcriptional promoter (5′HAM) and terminator (3′HAM) sequences, Cas12a sequences from *L. bacterium* fused to an oomycete nuclear localization motif (PsNLS), a 75 nt polyA region, and the Cas12a direct repeat. The ampicillin and G-418 (nptII) resistance genes used for selection in *E. coli* and *Phytophthora* are also illustrated. (**c**) Region of the plasmid liberated by *Bsa*I digestion of the pSTU plasmids. (**d**) Fragment inserted into the plasmids. This contains two *Bsa*I overhangs, the Cas12a direct repeat, and the 23 nt gRNA; the series of Ns will be changed based on the target sequence

4. It is preferable for gRNAs to match exactly their chromosomal targets, so check for potential polymorphisms between alleles within your strain of *Phytophthora* that might impair Cas12a activity or confound assays for editing (*see* **Note 7**).

3.2 Constructing the Editing Vector

1. Design the insert for the editing vector (Fig. 1b). Each gRNA should be preceded by a Cas12a direct repeat (DR), and the entire fragment should contain *Bsa*I overhangs at each end. If using an gRNA array, a DR must be placed between each (e.g.,

forming *Bsa*I–DR–gRNA1–DR–gRNA2–*Bsa*I). Adding a DR 3′ to the gRNA is unnecessary as one is within the backbone of the pSTU plasmids. While the insert for cloning can be designed to include whole *Bsa*I sites at each terminus, we find it more convenient to design two oligonucleotides which when annealed will have single-stranded tails compatible with the left and right *Bsa*I sites of the vector (TTTT and TAAT, respectively; Fig. 1d).

2. Order both the upper and lower strands from a commercial provider, and resuspend each in TE buffer to 100 μM.
3. Anneal the two oligonucleotides. First, combine 10 μL of each in a single tube and add 1 μL of 0.5 M NaCl to 0.05 M. Then, heat at 95 °C for 2 min, and cool to 4 °C over about 45 min. This can be done in a thermal cycler with a ramp program, or by heating the tube in a heat block which is then placed on the benchtop to cool to room temperature, followed by storage at 4 °C.
4. Digest the plasmid with tenfold excess of *Bsa*I. Following digestion, we recommend gel purification of the linearized plasmid to reduce any persisting undigested plasmid and to eliminate the stuffer fragment (Fig. 1c).
5. Mix the plasmid and insert in a 1:4 molar ratio and ligate. We normally perform ligations containing 1 μL of 10× T4 DNA ligase buffer with ATP, 1 μL (400 units) of T4 DNA ligase, a total of 100 ng of plasmid and insert, and water to 20 μL. This translates to about 98 ng of plasmid and 2 ng of insert for an insert with two gRNAs. Incubate overnight at 4 to 12 °C.
6. Mix 1 to 10 μL of the ligation reaction with 50 μL of competent *E. coli* cells (e.g., DH5α), incubate on ice for 30 min, and heat at 42 °C for 90 s (or 37 °C for 3 min). Then, add 400 μL of SOC or LB broth and place in a shaking incubator at 37 °C for 1 h. Plate aliquots on LB agar containing 100 μg/mL ampicillin or carbenicillin, and incubate 14–18 h at 37 °C until colonies are observed.
7. Identify clones containing the gRNA. Since the cloning procedure is highly efficient, we normally prepare plasmid DNA from three colonies and submit them for Sanger sequencing using primer 3′HAM34-R. This binds the *3′ Ham34* terminator allowing the sequence to read across the cloning site. Another option is to check the plasmids prior to sequencing for the absence of a *Bsa*I site, which should be lost upon cloning.
8. Prepare DNA for *Phytophthora* transformation using a scaled-up plasmid preparation method. In our hands, about 100 μg of DNA is typically more than sufficient for obtaining enough edited transformants, but this depends on the editing rate which varies depending on the gene and guide RNA (*see* **Note 8**).

3.3 Polyethylene Glycol-Mediated Protoplast Transformation

1. Prepare *P. infestans* cultures for protoplast transformation (*see* **Note 9**). Harvest sporangia from a 7- to 14-day-old stock culture by adding 10 mL of water followed by gentle rubbing with a spreader rod. Pipette off the liquid and count the sporangia with a hemocytometer. Spread about 5×10^4 sporangia on each of ten 150-mm plates of fresh rye-sucrose agar to establish the pre-inoculum culture. After allowing the liquid to absorb, invert the plates and grow for 7–9 d at 18 °C.
2. Isolate sporangia from the 150-mm plates by adding to each 15 mL of sterile H_2O, rubbing with a spreader rod, decanting off the liquid, and passing the resulting cloudy sporangia suspension through 50–70 μm nylon mesh or a cell strainer placed over a beaker. Add an equal volume of ALBA broth, mix gently, and count an aliquot using a hemocytometer. Dilute to 2.5×10^5 sporangia per mL by adding equal amounts of water and ALBA. Add about 150 mL of the sporangia mix to individual 1 L sterile flasks; avoid the temptation to put too much volume per flask as this may inhibit germination. Normally the ten 150-mm plates yield enough sporangia for four flasks.
3. Incubate the flasks at 18 °C without shaking for 24–36 h to allow the sporangia to germinate. The culture is normally ready to harvest when a thin mat of hyphae detaches from the bottom of the flask when swirled (*see* **Note 10**).
4. Collect the mat by pouring the culture through the nylon mesh. Any hyphae sticking to the flask can be scraped off using the tip of a pipette. The flow-through will contain mostly ungerminated sporangia; we recommend that this be incubated for another 24–36 h to yield tissue for a subsequent round of protoplasting, as this raises the efficiency of the procedure. Lift the hyphae from the nylon mesh using forceps, place in a 50-mL conical, and take note of the volume.
5. For every mL of loosely packed hyphae, prepare 3 mL of 10 mg/mL β-glucanase and 5 mg/mL cellulase in FPB. Typically, about 25 mL will be required. Sterilize using a syringe filter, placing the flow-through into a 50 mL conical tube.
6. Wash the hyphae from **step 4** once by adding 40 mL FPB, inverting, and passing through the nylon mesh or cell strainer. Lift out the washed mycelia using forceps and add to the tube with the protoplast enzymes (*see* **Note 11**).
7. Place the tube on its side on a rotary shaker. Shake at 50 rpm until protoplasting is about 90% complete, which should take about 25 min.
8. Filter through the cell strainer or nylon mesh, saving the flow-through in a beaker or 50 mL conical tube. Save a small amount to count the concentration. Pellet the protoplasts in a conical

tube by spinning at 700× *g* for 4 min in a swinging bucket rotor. While spinning, use a hemocytometer to count the starting protoplasts; anticipate losing about half in the following wash steps.

9. Carefully pour off the supernatant; the pellet may be soft. Use a pipette to remove any remaining drops of enzyme mix and resuspend the pellet in 30 mL FPB by stirring gently with a pipette. At this stage, thoroughly resuspending the pellet is not required and pipetting up and down is not recommended. Spin again at 700× *g* for 4 min.
10. Although DNA can be added directly to the protoplasts, we normally have better results by complexing the DNA with Lipofectin (*see* **Note 3**). It takes about 10 min for liposomes to form, so while the protoplasts are spinning, place 30 μg of plasmid DNA into a sterile polystyrene tube for each anticipated reaction (2×10^7 protoplasts for each 0.7 mL transformation); avoid polypropylene since this binds the liposomes. Add H_2O to a total of 40 μL, add 60 μL of Lipofectin, and gently pipette up and down or flick to mix. The mixture should turn cloudy. Reactions can be scaled up by increasing all components here and in subsequent steps in proportion.
11. Discard the supernatant from **step 9**, add 30 mL of 1:1 FPB: MT, and mix gently by stirring with a pipette; it is not necessary to thoroughly resuspend the pellet at this time. Spin at 700× *g* for 4 min.
12. Discard supernatant and add 30 mL MT supplemented with 10 mM $CaCl_2$. After thoroughly but gently resuspending the pellet, save a drop for counting on a hemocytometer, and spin the rest at 700× *g* for 4 min.
13. Discard the supernatant and resuspend pellet in enough MT supplemented with 10 mM $CaCl_2$ to have 2×10^7 of protoplasts per 0.7 mL reaction based on the count from Step 12. There should be enough protoplasts for about five reactions.
14. Add 0.7 mL of 50% PEG-MW 3350 containing 25 mM $CaCI_2$ and 10 mM Tris pH 7.5 to the protoplast-DNA mixture. Rotate or gently invert to mix, and incubate for 3–4 min.
15. Add clarified rye broth containing 1 M mannitol using the following stepwise procedure. First, add 2 mL of rye-mannitol to the protoplasts, and mix by inversion. After 30 s, add 4 mL of rye-mannitol, divide into two 50 mL tubes prefilled with 20 mL rye-mannitol, and invert. Lay the tube on its side and incubate at 18 to 25 °C for 18 to 30 h (*see* **Note 12**).
16. Check a drop from each tube for signs of regeneration, which is indicated by hyphae extending from the now-walled protoplasts. Using the entire tube, spin down at 700× *g* for 5 min

and decant the supernatant, leaving about 1 mL of free fluid in the tube. Gently resuspend the pellet in this residual liquid and spread 0.2 mL per 100-mm plate of rye-sucrose agar containing 8 μg/mL G-418. Incubate at 18 to 25 °C for 7 d, turning the plate upside down when the liquid is absorbed.

17. Start checking plates for colonies at day 6. These may continue to appear for up to 10 days while those appearing later are usually escapes resulting from G-418 breakdown. A successful transformation may yield 20–40 colonies per 2×10^7 of protoplasts. Transfer the colonies to fresh plates containing 8 μg/mL G-418 while they are still well-separated. If subsequent studies indicate that editing has occurred, G-418 does not need to be included in future cultures.

3.4 Extracting DNA from Phytophthora Transformants (See Note 13)

1. While colonies can be checked for editing on the original selection plates, we usually test them after being transferred to fresh plates.
2. Scrape a 1 cm^2 patch of hyphae from each culture, immerse in 0.3 mL extraction buffer in a 1.5 mL microcentrifuge tube, and place in a boiling water bath for 5 min.
3. Add 0.3 mL 1:1 phenol:chloroform. Vortex at high speed for 5 min, then spin for 5 min at 12,000× *g* or higher.
4. Pipette off 0.25 mL of the aqueous phase, place into a new 1.5 mL tube, and add 0.75 vol (0.187 mL) of 100% isopropanol. Mix well and spin for 10 min at 12,000× *g* or higher.
5. Wash the pellet by decanting the supernatant, adding 0.2 mL of 70% ethanol, spinning for 2 min, removing the supernatant, and air-drying the pellet for about 15 min.
6. Dissolve the DNA pellet in 30 μL of Low TE. This can be facilitated by heating at 65 °C for 5 min.

3.5 Analysis of Target Region

1. Obtain PCR primers flanking the gRNA target(s). We recommend using primers between 19 and 22 nt in size that yield an amplicon smaller than 800 nt, spaced at least 75 nt from each target.
2. For PCR, add 1 μL of the genomic DNA from Subheading 3.4 to a 25 μL reaction containing 1x PCR buffer, 1 mM $MgCl_2$, 200 μM dNTPs, 1 unit of *Taq* polymerase, and 0.2 μM of each primer. We normally perform 35-cycle reactions that include a 30 s denaturation step at 95 °C, 30 s of annealing at 50–60 °C depending on the primer, and 1 min of extension at 72 °C.
3. Visualize the products on a 1% agarose gel with a DNA stain. A single band sized differently than the unedited target will indicate biallelic editing resulting from NHEJ or monoallelic editing followed by gene conversion (Fig. 2a). A mixture of normal

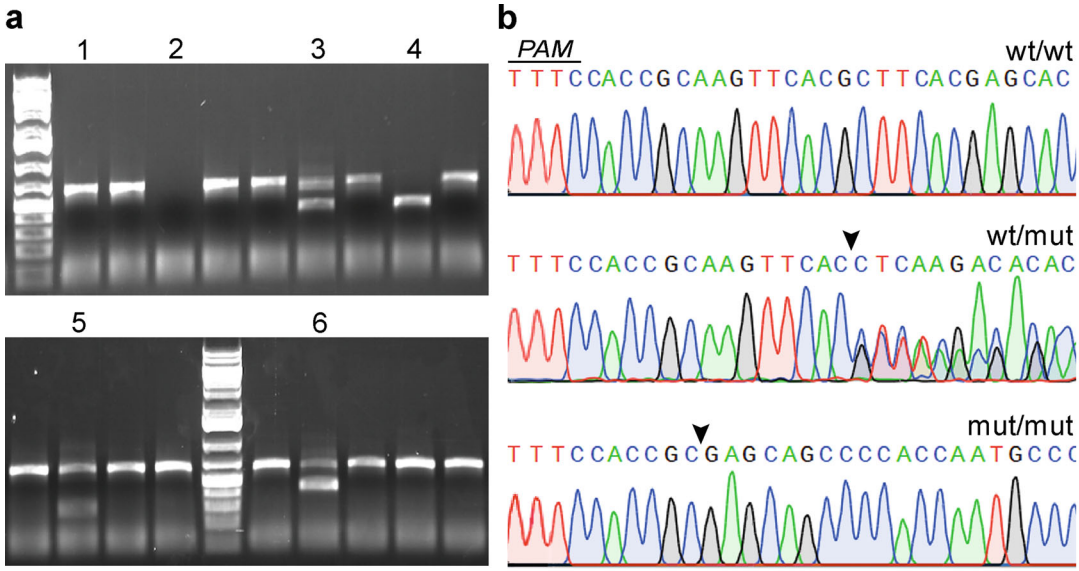

Fig. 2 Possible outcomes of editing. (**a**) PCR of target region in transformants. The lane labelled 1 is a representative unedited strain. Lane 2 is a strain in which a large deletion removed one or both primer sites; this would need to be re-screened with other primers to determine the precise nature of the editing event. Lane 3 is a heterozygote in which a monoallelic deletion resulted in two bands of equal intensity representing the unedited and edited alleles, respectively. Lane 4 is a biallelic edited strain; this possibly resulted from editing of one allele followed by gene conversion. Lane 5 is a likely heterokaryon containing both edited and unedited nuclei. The dominance of the wild-type band suggests heterokaryosis rather than heterozygosity. Lane 6, another possible heterokaryon in which the edited allele is stronger; further experimentation would be required to determine if this results from heterokaryosis or preferential amplification of the smaller band. (**b**) Sequencing chromatograms of transformants, starting from the PAM. The arrowheads indicate the 5′ junctions of the edited regions. Shown are an unedited strain (wt/wt), one in which a single allele was edited based on the presence of double peaks (wt/mut), and a strain containing two identical edited alleles (mut/mut)

and altered bands signals monoallelic editing. If the band ratio diverges much from 1:1, editing may have occurred after the first nuclear division, resulting in a heterokaryon. An absence of a band means either a large deletion or problems with DNA extraction or PCR (*see* **Note 14**).

4. Submit samples for Sanger sequencing (*see* **Notes 6** and **15**). This is done since small indels may not be evident from gel analysis, plus the results will allow the indel to be sized precisely to check for a frameshift that may block protein function. Potential outcomes are illustrated in Fig. 2b.
5. Heterokaryons containing a mixture of edited and unedited nuclei can often be converted to biallelic edited strains by single-zoospore (single nuclear) purification. This is also recommended for strains that will be used for experimental analyses such as phenotyping. This is achieved by isolating sporangia as in Subheading 3.3, **step 1** incubating them for

2 h at 10 °C to stimulate the release of zoospores, separating zoospores from sporangia by filtration through 15 μm mesh, and plating the zoospores at low density on selective media. Examination using a dissecting microscope can help identify incipient zoospore-derived colonies.

3.6 Protein Tagging by HDR

1. Plan the overall strategy by identifying the desired tag and whether the fusion should be N-terminal, C-terminal, or internal to the protein of interest. Illustrated in Fig. 3 is a strategy for adding a tag to the C-terminus (*see* **Note 16**).
2. Using the criteria in Subheading 3.1, design a gRNA that is preferably within 50 bp of the desired insertion site (*see* **Note 17**).
3. Once gRNA(s) are chosen, they should be synthesized and cloned into the Cas12a plasmid as in Subheading 3.2.
4. Design the HDR repair template, i.e., the donor DNA. This needs to include the tag (e.g., the fluorescent protein or epitope) flanked by homology arms that match the chromosomal target. We have had success with homology arms as small as 100 nt and as large as 500 nt. Since the optimal length of the homology arm may vary with the chromosome target and the distance between the Cas12a cut site and the desired location of recombination, we normally take a conservative approach of using arms between 500 and 1000 nt. The use of large arms was recommended in a prior study in *P. sojae* [2].

A short linker (~20 amino acids) should be provided to connect the tag with the protein of interest. The PAM or gRNA sequences within the donor should be altered to prevent re-cleavage by Cas12a, using silent point mutations if within the coding sequence (*see* **Note 18**).

5. Prepare the donor template with the following structure: left homology arm–tag–right homology arm. This is illustrated in Fig. 3 for a C-terminal mScarlet tag. The donor DNA is usually constructed in a general plasmid such as pBluescript using synthesized DNA as in Subheading 3.2, or by a ligation-independent long homology-based cloning method [27]. For HDR, we normally linearize the donor using one of the following three strategies. Option 1 is to linearize the plasmid prior to transformation by digestion with a restriction enzyme flanking one or both homology arms. Option 2 is to generate the linear DNA by PCR using primers binding the ends of the two homology arms. Option 3 is to construct a donor plasmid in which gRNA-PAM recognition sequences (the same as in **step 2**) are on the 5′ and 3′ ends of the homology arms, as shown in Fig. 3c. The latter approach relies on cleavage of the donor DNA in vivo during transformation (*see* **Note 19**).

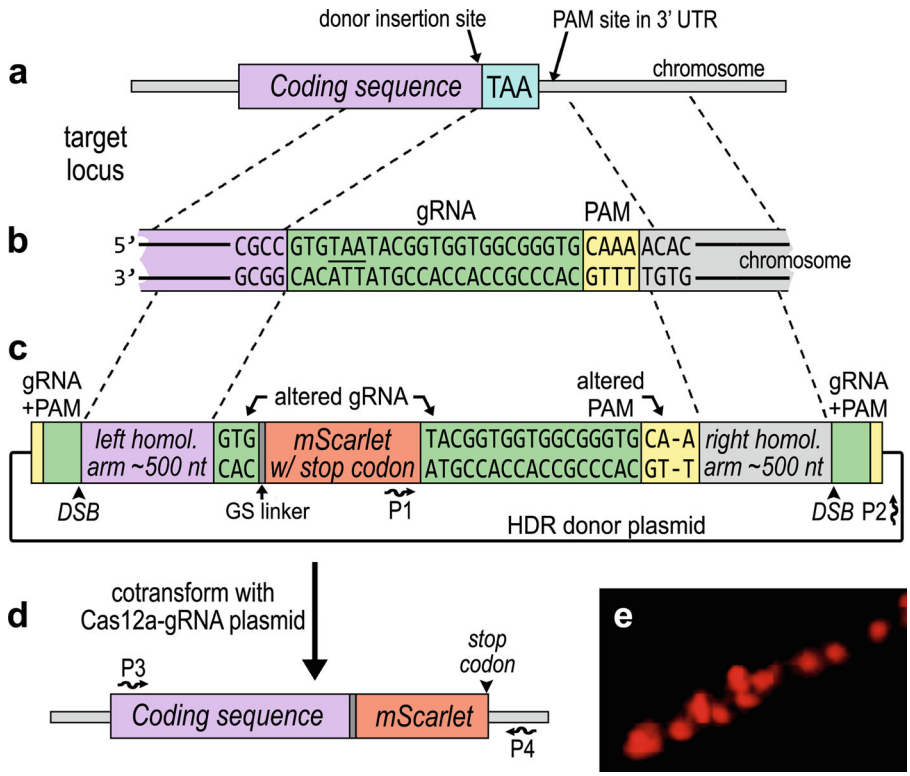

Fig. 3 Outline of C-terminal HDR tagging using mScarlet. (**a**) Schematic of a chromosomal target, indicating the desired insertion site 5′ of the stop codon and a PAM in the 3′ UTR. Having the gRNA within 50 nt of the insertion site is recommended to maximize HDR. (**b**) Closeup of the targeted region showing representative PAM and gRNA sequences. In this example, the stop codon is within the gRNA site (underlined), but this is not required for tagging since HDR results mostly from recombination within the homology arms, distal from the gRNA. (**c**) Diagram of the donor plasmid. This contains at least 500 nt of left and right homology arms located 5′ and 3′ of the insertion site, respectively; PAM and gRNA sequences added outside the homology arms to cause double-strand breaks (DSB) during transformation; a linker fused upstream of mScarlet (GS); a stop codon at the 3′ end of mScarlet; and an altered PAM to prevent continued editing by Cas12a after HDR. The placement of mScarlet within the gRNA site will also block continued cleavage, although altering both the PAM and gRNA regions is unnecessary. P1 and P2 represent PCR primers used to test for plasmid integration at random sites; HDR should result in no amplification since P2 binds to plasmid DNA outside the homology arm. (**d**) Structure of the chromosomal locus structure after HDR. P3 and P4 represent PCR primers used to help confirm in-frame incorporation of mScarlet and test if one or both alleles were tagged. These primers should reside distal to the homology arms. (**e**) Hyphal tip from a transformant in which a transcription factor was tagged with mScarlet. The protein localized to nuclei as expected

6. After obtaining sufficient DNA for transformation, co-transform *P. infestans* with the Cas12a-gRNA plasmid and the HDR donor plasmid (or linear DNA) in a 1:2.5 molar ratio. This is achieved as in Subheading 3.3 using a total of 35 to 50 μg DNA. A molar excess of donor template should raise the number of HDR events, although this may come at the cost of more off-target integrations.

7. Select transformants on rye containing G-418; a marker for the donor DNA is not used.
8. Screen transformants for incorporation of the tag. If using a fluorescent tag, we normally first test for its expression by microscopy (*see* **Note 20**).
9. Confirm the nature of the integration event by extracting genomic DNA, followed by PCR and Sanger sequencing as in Subheadings 3.4 and 3.5. This is needed to distinguish between precisely edited clones and off-target integrations, and between mono- and biallelic editing. For downstream studies, we recommend selecting transformants bearing only one edited allele to minimize any effects of tagging on protein function. Suggested binding sites for the primers are shown in Fig. 3c and d; several primer pairs may be needed depending on the size of the genes and homology arms.
10. Single-zoospore purification of successfully tagged transformants is recommended to isolate a pure strain containing the desired heterozygous or homozygous knock-ins.

4 Notes

1. pSTUC1 is a derivative of Cas12a, which includes amino acid changes that allow editing to function better over a broad temperature range [28]. We recommend using this variant for species like *P. infestans* that grow at cooler temperatures, e.g., 18–22 °C. We have not tested whether this variant is superior for species of *Phytophthora* having higher optimal growth temperatures, e.g., 28 °C or above.
2. Some published editing protocols using Cas9 include a step for phosphorylating the oligonucleotides, but this is not required unless the vector has been dephosphorylated to block self-ligation. The latter scenario should not occur with our vectors since their two *Bsa*I sites do not generate cohesive ends.
3. We normally use a *Trichoderma* cellulase purchased from MilliporeSigma (catalog number C8546), but most other cellulases work equally well. We have used several β-glucanases with success but found that the most effective and economical choices are products developed for clarifying wines, such as Vinoflow NCE (Novozymes) and Extralyse (Laffort).
4. We have found that Lipofectin increases the number of transformants by two to threefold, but this can be omitted.
5. The drug concentration may need to be varied depending on the strain, species, or lot of chemical. We suggest performing a preliminary experiment to identify the minimal concentration that blocks growth. Other types of media may also be suitable

for species besides *P. infestans*. We have also constructed editing vectors using hygromycin as the selectable marker, as G-418 may not be suitable for all oomycetes.

6. PCR reactions must be purified prior to DNA sequencing to remove primers and nucleotide triphosphates. This can be done using a PCR clean-up column or by treatment with exonuclease I and (shrimp) alkaline phosphatase. We find the latter to be more convenient. Both enzymes are available from New England Biolabs, or packaged together in the ExoSAP-IT kit from ThermoFisher or the Exo-Alp PCR Cleanup Mix kit from AdvancedSeq.
7. The gRNA and target should match perfectly within the seed region, which for Cas12a is the first 5–6 bases after the PAM. Up to two mismatches elsewhere often do not severely impair editing [29, 30]. Nevertheless, we aim for monomorphic sites to maximize editing efficiency. Monomorphism within the binding sites for PCR primers used to test for editing is also important to ensure that both alleles will be amplified. If the gRNAs were designed using a genome from a strain other than the one being transformed, the occurrence of inter-strain polymorphisms should also be considered. Ideally, such issues can be addressed by examining a phased genome assembly of the strain that will be edited. Alternatively, one can search unassembled DNA sequence reads from Illumina or other technologies; such data can also be used to test for diploidy considering that knocking out genes in polyploids may be more challenging [8]. Data about polymorphisms might be obtained by sequencing PCR products from the genomic target, but this assumes that the primers would bind both alleles. RNA reads may also be useful, although we have observed cases where the alternate alleles of a gene were transcribed at very dissonant levels [3].
8. To ensure maximum yield, we recommend streaking out a fresh plate of a bacterial colony containing the vector. Pick a colony and grow in 2 mL of LB or TB both containing 100 μg/mL carbenicillin to exponential phase (4–8 h) at 37 °C with 250 rpm; this antibiotic persists longer in media than ampicillin. Inoculate 0.1% volume (250 μL) into 250 mL of TB containing 100 μg/mL carbenicillin in a 1 L flask. Incubate at 37 °C and 250 rpm for no longer than 12 h prior to DNA extraction; the total yield is normally 0.3 to 1.2 mg.
9. Several other techniques exist for transformation, including zoospore electroporation and *Agrobacterium*-mediated transformation [11, 12]. We usually employ the protoplast method for editing since it can yield the most transformants, but good evidence that the resulting transformants exhibit higher rates of editing is lacking.

10. Obtaining young tissue for protoplasting is important. Older cultures will digest poorly and yield many anucleate protoplasts, which fruitlessly soak up DNA. Our protocol is optimized for *P. infestans* isolate 1306 to achieve more synchronous germination of sporangia, yielding more young hyphae and hence, more transformants. The timepoints may need to be altered for other strains, and in some cases, it may be useful to test other approaches for obtaining young hyphae such as by germinating zoospores in media.
11. It is important to move quickly through the succeeding steps to minimize the time that the tissue and protoplasts are not in media. To help accomplish this, in the next step we collect the protoplasts before the hyphae is 100% digested.
12. Although our previously published transformation procedures describe incubating protoplasts at 18 °C following DNA treatment, for editing we recommend regenerating the protoplasts for one day at 25 °C prior to spreading on agar media. This is above the optimal growth temperature for *P. infestans,* but increases the frequency of editing [28]. After plating on agar media, cultures can be shifted to 18–20 °C since most editing events appear to occur within a day after treating the protoplasts with DNA. We recommend spreading the regenerated tissue on agar media after no more than 24 h of growth in broth, since longer incubation times will result in matted hyphae that are difficult to distribute on the plates. We also recommend including an antifungal and antibacterial in the regeneration broth, such as 10,000 units/mL of nystatin and 50 μg/ml penicillin G.
13. We have found that this method yields more reliable results in PCR than DNA from column-based kits.
14. The editing rate will vary depending on the gene, gRNA, and transformation reaction. While we have observed NHEJ in up to 75% of transformants in some experiments, a frequency of 15 to 20% is more typical. Cases in which no editing or only non-frameshift mutations are observed may signal that the target is an essential gene.
15. DNA for sequencing can be isolated directly from the PCR sample. If nonspecific bands are present, we advise gel purification or, better yet, repeating the amplification with more specific primers. Otherwise, the minor bands may yield peaks in chromatograms that would erroneously suggest editing.
16. The location for the tag should be tailored to the protein. For example, a tag should not be placed upstream of a gene for a secreted protein as this may interfere with localization. If a fluorescent reporter is used, choose one that is photostable, bright, and imageable with equipment available to your lab.

When expressed as a fusion protein, monomeric tags such as mScarlet have less tendency to induce toxicity than proteins such as GFP which may dimerize [31, 32].

17. A 50 nt window is suggested since the frequency of HDR may decline with increasing distance from the desired insertion site [33]. Note that the double-strand break generated by Cas12a serves only as the site to initiate repair. The final exchange points between chromosome and donor will be within the homology arms, potentially distant from the double-strand break. Nevertheless, placing the gRNA in a non-coding region is preferred to avoid altering the protein's sequence outside of the tag.
18. Linkers are added to minimize the risk of sterically hindering protein function. Their optimal sequence depends on the nature of the tag and its desired application [34]. Nevertheless, most linkers consist of amino acids such as glycine and serine that provide flexibility [35]; we had success with $(GGGGS)_2$. Besides adding a linker, remember to design the donor to eliminate unwanted stop codons.
19. Although a linear donor is not essential for HDR, most studies have reported higher efficiencies with linear versus circular (plasmid) donors [36]. We have usually generated a linear donor using Option 3 due to reports that this improved HDR in other systems [37]. The theory is that the simultaneous activation of the target site and in vivo linearization of the donor construct synchronizes the demand and supply of homologous sequences, thus increasing HDR. However, data comparing HDR efficiencies in *Phytophthora* using circular, single-cut linearized, or double-cut linearized templates are not yet available.
20. Alternatively, Western blot analysis can be performed on cell lysates using antibodies against the fluorescent reporter or epitope tag. This will also confirm if the fusion protein has the expected size.

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